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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Andrew Goodearl et al.	Art Unit	:	1818
Serial No.	:	08/736,019	Examiner	:	S. Gucker
Filed	:	October 22, 1996			
Title	:	GLIAL MITOGENIC FACTORS, THEIR PREPARATION AND USE			

Assistant Commissioner of Patents and Trademarks
Washington, DC 20231DECLARATION OF MARK MARCHIONNI, Ph.D. 37 CFR § 1.132

I, Mark Marchionni, Ph.D., declare as follows.

1. I received my Ph.D. in Biology in 1980 from Kansas State University. I completed a post-doctoral fellowship, from 1981 until 1987, with Walter Gilbert, Ph.D. at Harvard University. My fellowship research related to gene evolution and molecular biology. I have authored over 20 peer-reviewed research publications in molecular and cellular biology. Since 1987 I have been employed as a scientist by Cambridge Neuroscience Inc. From 1987-1996 I held the position of Group Leader in Molecular Biology, and since October 1996, I have held the position of Senior Scientist. I am also a

co-inventor of the above- referenced patent application.

2. I am the co-author of Canoll, P.D., Musacchio, J.M., Hardy, R., Reynolds, R., Marchionni, M.A., and Saltzer, J.L. (1996). "GGF/Neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors." *Cell*, 17:229-243 (hereafter "Canoll") (enclosed). Canoll is published in a peer-reviewed scientific journal.
3. In Canoll et al., it is clearly demonstrated that recombinant human GGF2 and recombinant heregulin $\beta 1$ (a GGF/p185erbB2 ligand gene product), both of which have EGF-like domains and are ligands for the p185/erbB2 receptor, are mitogenic for multiple types of glial cells of the central nervous system, specifically, pro-oligodendrocytes, oligodendrocytes, and type-2 astrocytes. Mitogenesis was measured by increased BrdU incorporation into the nuclei of treated cells. This general technique for measuring mitogenesis was provided in the present application (Example 3, pages 44-45), and the relevant primary glial cells were isolated using methods known in 1990 or earlier.
4. GGF2 and heregulin $\beta 1$ show similar efficacy in inducing glial cell mitogenesis, which suggests that the effects of GGF2 are not isoform-specific. This is consistent with previous reports that the epidermal growth factor-like domain is sufficient to mediate the mitogenic effect of GGF2 family members.
5. I conclude from the results described above, and from other similar results, that polypeptides having epidermal growth factor-like domains encoded by the

GGF2/p185erbB2 ligand gene are mitogenic for a variety of glial cell subtypes, including those of the central nervous system.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date:

Sept 3 1998

Mark Marchionni

Mark Marchionni, Ph.D.

GGF/neuregulin Is a Neuronal Signal That Promotes the Proliferation and Survival and Inhibits the Differentiation of Oligodendrocyte Progenitors

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Summary

We show that GGF/neuregulin is a mitogen for pro-oligodendrocytes (O4+/O1- cells), oligodendrocytes (O4+/O1+ cells), and type-2 astrocytes. Heregulin B1, another neuregulin isoform, is also mitogenic. The proliferative effect of glial growth factor (GGF) does not require, but is greatly potentiated by, serum factors. GGF also promotes the survival of pro-oligodendrocytes under serum-free conditions. High levels of GGF reversibly inhibit the differentiation and lineage commitment of oligodendrocyte progenitors and, in differentiated cultures, result in loss of O1 and myelin basic protein expression. All three erbB receptors are expressed by progenitors and are activated by GGF; the relative abundance of these receptors changes during differentiation. Finally, cortical neurons release a soluble mitogen for pro-oligodendrocytes that is specifically blocked by antibodies to GGF. These results implicate the neuregulins in the neuronal regulation of oligodendrocyte progenitor proliferation, survival, and differentiation.

Introduction

Oligodendrocyte progenitors arise from the subventricular zone (SVZ) and migrate out from there into developing white matter, differentiating and proliferating en route (Altman, 1966; Goldman, 1992). From the time these cells migrate out from the SVZ and come to reside in white matter tracts where they myelinate axons, they are almost continually in a neuronal environment. Indeed, a variety of studies indicate that neurons profoundly influence the survival, proliferation, and differentiation of cells of the oligodendrocyte lineage (reviewed by Barres and Raff, 1994; Hardy and Reynolds, 1993a).

Transection of the optic nerve during development results in a severe reduction in the numbers of oligodendrocytes, but not astrocytes, suggesting a dependence of developing oligodendroglia on neuronal survival factors (David et al., 1984; Fulcrand and Privat, 1977), a finding confirmed in coculture studies (Levine, 1989; Shaw et al., 1996). Transection of the adult optic nerve results in a substantial reduction in the expression of myelin-related genes by oligodendrocytes (Kidd et al., 1990; McPhilemy et al., 1990), indicating that later in development axons regulate oligodendrocyte differentiation. Finally, neurons and neuronal extracts are known to provide a mitogenic signal for both immature and mature oligodendrocytes (Chen and DeVries, 1989; Edgar and Pfeiffer, 1985; Wood and Bunge, 1986) that depends, in part, on the electrical activity of the axon (Barres and Raff, 1993).

Characterization of the factors that regulate oligodendrocyte proliferation, differentiation, and survival has been facilitated by the ability to generate and analyze cells at different stages of the oligodendrocyte lineage. Cells at different stages of this lineage can be distinguished, in turn, by their morphology and by their expression of different antigenic markers (Pfeiffer et al., 1993; Raff, 1989). An early cell in the lineage, the O-2A cell, is recognizable by its bipolar morphology, by its expression of a ganglioside recognized by the A2B5 monoclonal antibody, and by its failure to express markers of either mature astrocytes, such as glial fibrillary acidic protein (GFAP), or of oligodendrocytes, including galactocerebroside (GalC). While the normal fate of these progenitor cells in vivo is probably to develop into oligodendrocytes (Monteros et al., 1993), some plasticity exists that is dependent on unknown environmental signals (Levison and Goldman, 1993). In vitro, O-2A cells demonstrate a great deal of phenotypic plasticity, differentiating into either oligodendrocytes or type-2 astrocytes depending on culture conditions (Raff, 1989). When cultured in the presence of low concentrations of serum, these cells progressively differentiate along the oligodendrocyte lineage, sequentially expressing glycolipids recognized by the O4 antibody and the O1 antibody (which reacts with GalC) and then myelin-specific proteins, including myelin basic protein (MBP) (Pfeiffer et al., 1993). In contrast, culturing cells in the presence of high concentrations of serum, or in the presence of certain growth factors such as ciliary neurotrophic factor, favors the expression of astrocytic markers, notably GFAP (reviewed by Raff, 1989). Thus, cultures greatly enriched in O-2A cells (A2B5+/O4-), pro-oligodendrocytes (O4+/O1-), oligodendrocytes (O4+/O1+), or type-2 astrocytes (GFAP+) may be generated by varying the conditions of cell culture.

By using such culture models, several candidate mitogens and survival factors have been identified that are effective at different stages of the oligodendrocyte lineage. Among the best-characterized are platelet-derived growth factor (PDGF), which is mitogenic for early cells in the oligodendrocyte lineage (i.e., A2B5+/O4-) (Noble et al., 1988; Richardson et al., 1988), and

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basic FGF (bFGF), which is a mitogen for more differentiated oligodendrocytes (Eccleston and Silberberg, 1984; Fressinaud et al., 1995; Saneto and Vellis, 1985). When used in combination, these two growth factors cooperate to inhibit the differentiation and maintain the proliferation of oligodendrocyte progenitors (Bögler et al., 1990; Collarini et al., 1992; McKinnon et al., 1990). PDGF and, possibly, bFGF also promote the survival of oligodendrocyte progenitors (Barres et al., 1992; Yasuda et al., 1995). Other factors that promote proliferation or survival (or both) of oligodendrocyte progenitors include neurotrophin-3 (Barres et al., 1993) and insulin-like growth factor (McMorris and Dubois-Dalcq, 1988; for a review, see Barres and Raff, 1994). All of these factors are released by astrocytes; bFGF and PDGF are expressed and released by neurons as well (Dutly and Schwab, 1991; Hardy and Reynolds, 1993a; Pettman et al., 1986; Yeh et al., 1991).

Neurons and a neuronal cell line also release a mitogen(s) that is distinct from either PDGF or bFGF (Gard and Pfeiffer, 1990; Hardy and Reynolds, 1993b; Hunter and Bottenstein, 1991; Levine, 1989). We considered whether the neuregulins might correspond to this unidentified mitogenic activity. The neuregulins are a family of soluble and transmembrane protein isoforms encoded by a single gene that includes the glial growth factors (GGFs), the neu differentiation factor (NDF), the heregulins, and the acetylcholine receptor-inducing activity (ARIA) (Marchionni et al., 1993; Peles and Yarden, 1993). These proteins mediate their effects by binding to and signaling via a family of receptor tyrosine kinases comprised of erbB2, erbB3, and erbB4 (reviewed by Carraway and Burden, 1995). Although initially purified as a potential ligand for erbB2, recent studies suggest that neuregulins bind to erbB3 and erbB4, which heterodimerize in various combinations with each other, and with erbB2 and the epidermal growth factor receptor to effect subsequent signaling.

We now report that a soluble recombinant form of human neuregulin, corresponding to glial growth factor 2 (GGF2), is a potent mitogen for oligodendrocytes and their progenitors. GGF also blocks their differentiation at the O4+ stage and enhances the survival of these cells. In addition, we demonstrate that the mitogenic effect of cortical neuron-conditioned media (CCM) on oligodendrocyte progenitors is inhibited by antibodies to GGF. Finally, neuregulin receptors are shown to be expressed in cells at various stages of the lineage and can be activated by exposure to GGF. These results indicate that the neuregulins are physiologic mitogens for oligodendrocyte progenitors and have potent effects on their survival and differentiation.

Results

GGF Is a Potent Mitogen with Pleiotropic Effects on Cells of the Oligodendrocyte Lineage

To investigate the potential effects of GGF on cells of the oligodendrocyte lineage, we first established cultures of oligodendrocyte progenitors from neonatal rat cortex essentially as described (Louis et al., 1992). Cells were expanded by supplementing the media with B104 conditioned media. Cells in the supplemented media had a bipolar morphology and remained highly proliferative, and >95% of the cells expressed vimentin and A2B5, but not O4, O1, or GFAP. When switched to a defined media to which 0.5% serum was added to enhance survival (DM*), the cells rapidly acquired a multipolar morphology and began to express the O4 antigen. After several days, the cells developed a complex network of processes and began to express the O1 antigen. By 3 days in DM*, essentially all cells were O4+ and over 50% were O1+ and MBP+. Cell differentiation coincided with a substantial reduction in the proliferative rate. The labeling index of cells grown in B104 conditioned media was greater than 50% after a 4 hr pulse of bromodeoxyuridine (BrDU) but decreased to less than 5% when cells were grown in DM* for 3 days.

To characterize the effects of GGF, cells grown in DM* for 3 days were treated with 200 ng/ml GGF for 16 hr, the last 4 hr in the presence of BrDU. Cells were then fixed and stained for O4, O1, and BrDU. The results, shown in Figure 1, demonstrate that GGF had a number of dramatic effects. After GGF treatment, all cells remained O4+ (Figure 1B), but most treated cells had a less complex morphology than control cells (Figure 1A), with fewer and flatter processes suggestive of a less mature phenotype. Consistent with this change in morphology, significantly fewer O1+ cells were present after GGF treatment compared with controls (Figures 1C and 1D). Finally, there was a substantial increase in the number of BrDU-positive nuclei in the treated compared with the control cells (Figures 1E and 1F). We have quantitated these effects over a range of GGF concentrations. The labeling index was determined separately for O4+ and O1+ cells. In each case, the labeling index increased with higher concentrations of GGF (Figure 1G). This was paralleled by the loss in O1 expression (Figure 1H). We consider first the mitogenic response to the neuregulins.

GGF is a potent mitogen for O4+ cells. The labeling index of the O4+ cells increased significantly with concentrations of GGF as low as 0.2 ng/ml and reached a plateau of ~40% at concentrations of 200 ng/ml or greater (Figure 1G); a half maximal response was observed with concentrations of GGF of about 20 ng/ml.

(Figure 1 legend continued)

cells in the field. O4 and O1 staining was visualized with a rhodamine-conjugated antibody, BrDU with a fluorescein-conjugated antibody. Separate fields from a single experiment are shown that are representative of the staining pattern of each antibody. Scale bar, 50 μ m (A-D). (G) O4+ progenitors and O1+ oligodendrocytes exhibit different mitogenic responses to GGF. The labeling index was determined for O4+ and O1+ cells after adding different concentrations of GGF for 16 hr; BrDU was added for the last 4 hr. Each point represents the mean \pm SEM from two independent experiments. (H) Loss of O1 expression with increasing concentrations of GGF. The percentage of O1+ cells was determined from cultures that were treated with increasing concentrations of GGF (described above in [G]). Each point represents the mean \pm SEM from two independent experiments.

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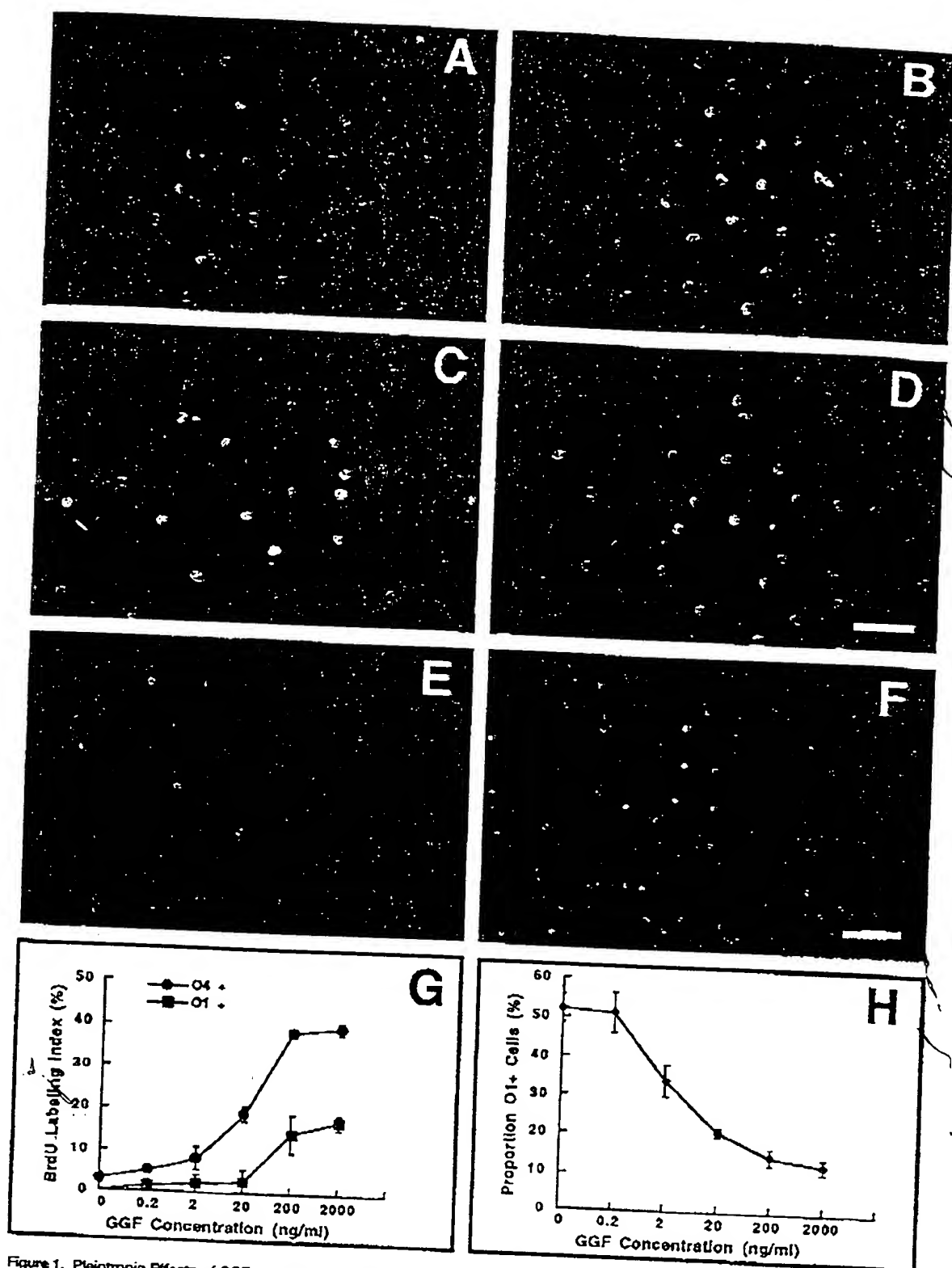


Figure 1. Pleiotropic Effects of GGF on Cells in the Oligodendrocyte Lineage

(A)-(F) Effects of GGF on cells of the oligodendrocyte lineage. Cells were allowed to differentiate in DM⁺ for 3 days; cultures were then fed with DM⁺ without (A, C, and E) or with 200 ng/ml GGF (B, D, and F) for 16 hr. In each case, BrdU was added during the last 4 hr. Cultures were fixed and stained for O4 (A and B), for O1 (C and D), or for BrdU (E and F). (A)-(D) were counterstained with Hoechst dye to identify all

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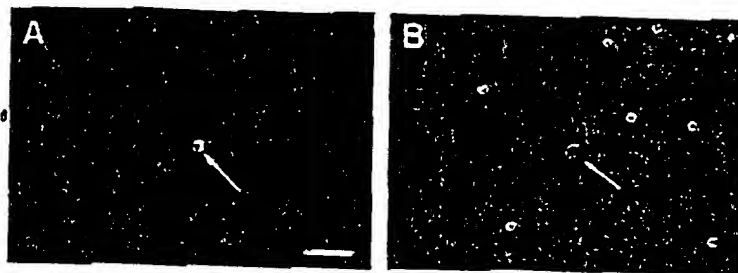


Figure 2. GGF Is Mitogenic for MBP+ Oligodendrocytes

Cells maintained in DM* for 3 days were treated with 200 ng/ml GGF for 16 hr; BrdU was added during the last 4 hr of GGF treatment. Cells were immediately fixed and stained for MBP and BrdU and counterstained with Hoechst dye to identify all cells in the field. (A) shows the Hoechst-stained cells, including an MBP+ oligodendrocyte. (B) demonstrates multiple BrdU+ nuclei in the same field. The nucleus of the oligodendrocyte is BrdU+ and is indicated by an arrow in each panel. Scale bar, 25 μ m.

(~0.5 nM). The mitogenic effect is not specific for the GGF isoform, as heregulin β 1, another neurotrophin isoform expressed in the CNS, also has proliferative effects. O4+ cells treated for 16 hr with conditioned media, with or without heregulin β 1 (Jo et al., 1995), displayed a comparably robust mitogenic response. Thus, cultures treated with control media had a labeling index of $3.4\% \pm 0.4\%$, whereas heregulin β 1-treated cultures had a labeling index of $33.3\% \pm 2.4\%$.

GGF is also mitogenic for O1+ cells, although they appear to be less responsive than the total population of O4+ cells. The labeling index of O1+ cells in the control cultures was 0%; it increased to nearly 5% with concentrations of GGF up to 20 ng/ml and increased to just above 15% at higher concentrations of GGF (200–2000 ng/ml). In separate experiments, a similar mitogenic effect on MBP+ cells was also observed, i.e., there were no MBP+/BrdU+ cells in controls versus $16.7\% \pm 4.3\%$ in the cultures treated with 200 ng/ml GGF. A representative example of an MBP+ cell that incorporated BrdU is shown in Figure 2. However, because GGF treatment may down-regulate the expression of these mature markers as described below, these studies may underestimate the actual mitogenic response of oligodendrocytes.

Indeed, one of the most striking effects of short-term treatment with GGF was a marked decrease in the number, and percentage, of O1+ cells from these more differentiated cultures. This decrease was dose dependent and inversely correlated with the mitogenic response to GGF (see Figures 1G and 1H). Treatment with 200 ng/ml GGF resulted in a dramatic reduction in the absolute number of O4+/O1+ cells from 26.5 ± 4.8 per high power field (HPF) in control cultures to 4.5 ± 0.7 per HPF; thus, the percentage decrease in O4+/O1+ cells is not due to a selective expansion of O4+/O1- cells by GGF. In separate experiments, a similar decrease in the absolute number of MBP+ cells was observed in GGF and heregulin β 1-treated cultures. These findings suggest that the loss of O1 and MBP expression reflects reversion of O1+ cells to the O1- stage or selective cytotoxicity of neurotrophins for O1+ cells (for example, see Muir and Compston, 1996). As total cell numbers increase during the period of GGF treatment and cells appear viable, we favor the former possibility. Further studies are underway to elucidate the precise mechanism involved.

Finally, to determine whether GGF was mitogenic for type-2 astrocytes, O-2A cells growing in media supplemented with B104 conditioned media were switched to media containing 20% fetal bovine serum (FBS). We

observed that 3 days later, over 50% of the cells expressed high levels of GFAP and developed a stellate morphology characteristic of type-2 astrocytes (Figure 3A). In parallel, the labeling index rapidly declined to $2.5\% \pm 0.7\%$ (Figure 3C). After treatment with GGF for 16 hr, there was a striking change in the morphology of the astrocytes with some cells exhibiting a few highly extended GFAP+ processes (Figure 3B). Of particular note, the labeling index of the total population of cells increased markedly to $14.5\% \pm 1.2\%$ (Figure 3D). Most of the BrdU+ cells were GFAP-. In separate experiments, type-2 astrocytes, identified by double labeling with GFAP, also demonstrated a modest but significant mitogenic response, with the labeling index increasing from 0% in the controls to 8% in the GGF-treated cultures.

GGF Has Direct Mitogenic Effects

We next examined whether the mitogenic activity of GGF was direct or whether GGF stimulated proliferation by inducing progenitor cells to release another growth factor that in turn increased proliferation. To this end, we first determined the neutralizing activity of an antibody to GGF. Preincubating GGF with this antibody prior to adding it to O4+ cells completely inhibited its mitogenic effect (Figure 4A); the same antibody did not inhibit the mitogenic effect of bFGF or PDGF, thereby demonstrating its specificity (data not shown). We next collected media conditioned by O4+ cells grown for 1 day with GGF (200 ng/ml) and added this media to cells directly or after preincubation with the GGF neutralizing antibody. Conditioned media from GGF-treated cells, when added directly, resulted in a 6-fold increase in the labeling index of the recipient O4+ cells after 16 hr (Figure 4B). The mitogenic effect of this conditioned media was completely inhibited by preincubation with the anti-GGF antibody. Incubation with an anti-PDGF antibody, which was used as an antibody control, had no effect on the mitogenic activity of the conditioned media. These results demonstrate that the mitogenic effect of GGF on O4+ cells is direct and does not result from the release of other mitogens into the conditioned media.

To determine whether the mitogenic activity of GGF was dependent on serum factors, oligodendrocyte progenitors were switched from media supplemented with B104 conditioned media to a defined media without any serum added. After 3 days, GGF was added to these cultures and the extent of proliferation was measured as above. In the absence of serum and GGF, the rate

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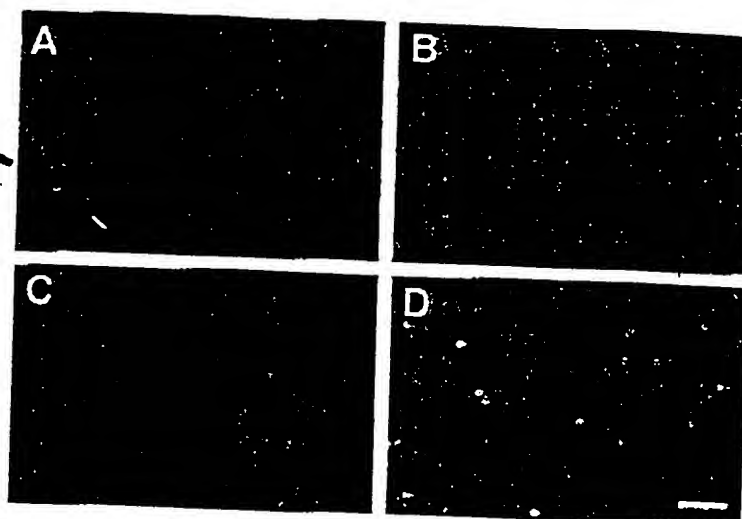


Figure 3. GGF Effects on Type-2 Astrocytes
Type-2 astrocyte-enriched cultures were established by growing cells for 3 days in media containing 20% FBS. Media was then added without (A and C) or with 200 ng/ml GGF (B and D) for 16 hr, the last 4 hr with BrDU. Cells were fixed and stained for GFAP expression (fluorescein secondary) and counterstained with a nuclear dye (A and B) or stained for BrDU (fluorescein antibody) as shown in (C) and (D). Separate fields from a single experiment are shown that are representative of the staining pattern of each antibody. Scale bar, 25 μ m.

of proliferation was markedly reduced, with a labeling index of $0.4\% \pm 0.2\%$ after a 4 hr incubation with BrDU. In contrast, in cultures treated with 200 ng/ml GGF, the rate of proliferation was significantly increased, with a labeling index of $7.1\% \pm 0.3\%$. These results indicate that the mitogenic effect of GGF on oligodendrocyte progenitors does not require serum, although it is greatly enhanced in the presence of low concentrations of serum.

GGF Promotes Oligodendrocyte Progenitor Survival

Survival of oligodendrocyte progenitors in vitro is dependent on the presence of a number of growth factors

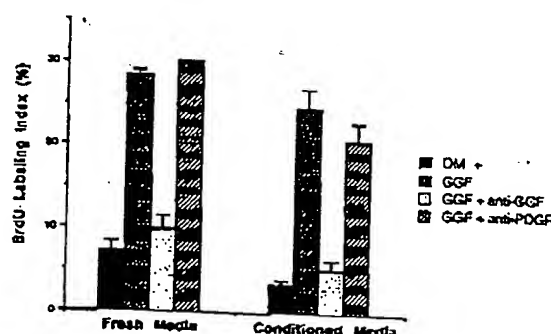


Figure 4. GGF Is a Direct Mitogen for O4+ Cells

Left: the mitogenic effect of GGF on O4+ cells is blocked by a neutralizing antibody. O4+ cells (3 days in DM*) were grown for 16 hr in DM* without GGF (control), with GGF, with GGF and an anti-PDGF antibody, or with GGF and an anti-GGF antibody; BrDU was added during the last 4 hr. Cells were fixed and the percentage of labeled nuclei was determined for each condition. Bars represent the mean \pm SEM of duplicate wells from two independent experiments.

Right: GGF is a direct mitogen. O4+ cells (3 days in DM*) were incubated for 16 hr with conditioned media from control cells or from cells treated with GGF (GGF conditioned media). The GGF conditioned media was added directly to recipient cells or added with anti-GGF or anti-PDGF neutralizing antibodies. A 4 hr pulse of BrDU was given and the labeling index determined. Bars represent the mean \pm SEM of duplicate wells from two independent experiments.

or low concentrations of serum (for example, see Barres et al., 1992). Consistent with earlier reports, we noted in the studies described above large numbers of dead cells in the control cultures following serum withdrawal. By contrast, GGF-treated cultures had notably fewer dead cells, strongly suggesting that GGF promotes the survival of oligodendrocyte progenitors. To directly examine whether GGF has survival effects for oligodendrocyte progenitors, cells were grown in DM* for 3 days then switched to a serum-free defined media with or without GGF. Survival was analyzed after an additional 12 or 24 hr. Initial experiments were done in N2 media and demonstrated that GGF strongly promoted oligodendrocyte progenitor survival as assessed by nuclear dye exclusion (data not shown). However, because the N2 media contains concentrations of insulin (5 μ g/ml) that enhance oligodendrocyte survival (Barres et al., 1992), these experiments were repeated in Dulbecco's modified Eagle's medium (DMEM) without any supplements. Similar effects on survival were apparent under these more stringent conditions (Figure 5). Thus, in the absence of GGF, ~50% of the cells were dead at 12 hr (Figure 5A), whereas at 200 ng/ml GGF only about 10% of the cells were dead. After 24 hr in DMEM, most of the cells were dead in the control cultures, whereas substantial numbers of viable cells remained in the treated cultures (data not shown). Consistent with this assay, the control wells had many more pyknotic cells in small aggregates than the GGF-treated wells; significantly fewer cells in the control cultures also remained attached after changing the media. The survival effects of GGF on O4+ cells were dose dependent with a half maximal response of ~2 ng/ml (Figure 5C).

Because of greatly improved cell survival in the presence of serum, all subsequent studies described below have been done in the presence of 0.5% serum, i.e., in DM*.

GGF Maintains Progenitor Cell Proliferation and Inhibits Differentiation

To determine whether the effects of GGF are sustained, or, like PDGF are limited to a few cell cycles (Raff et al., 1988), we treated O-2A cells with GGF chronically. We

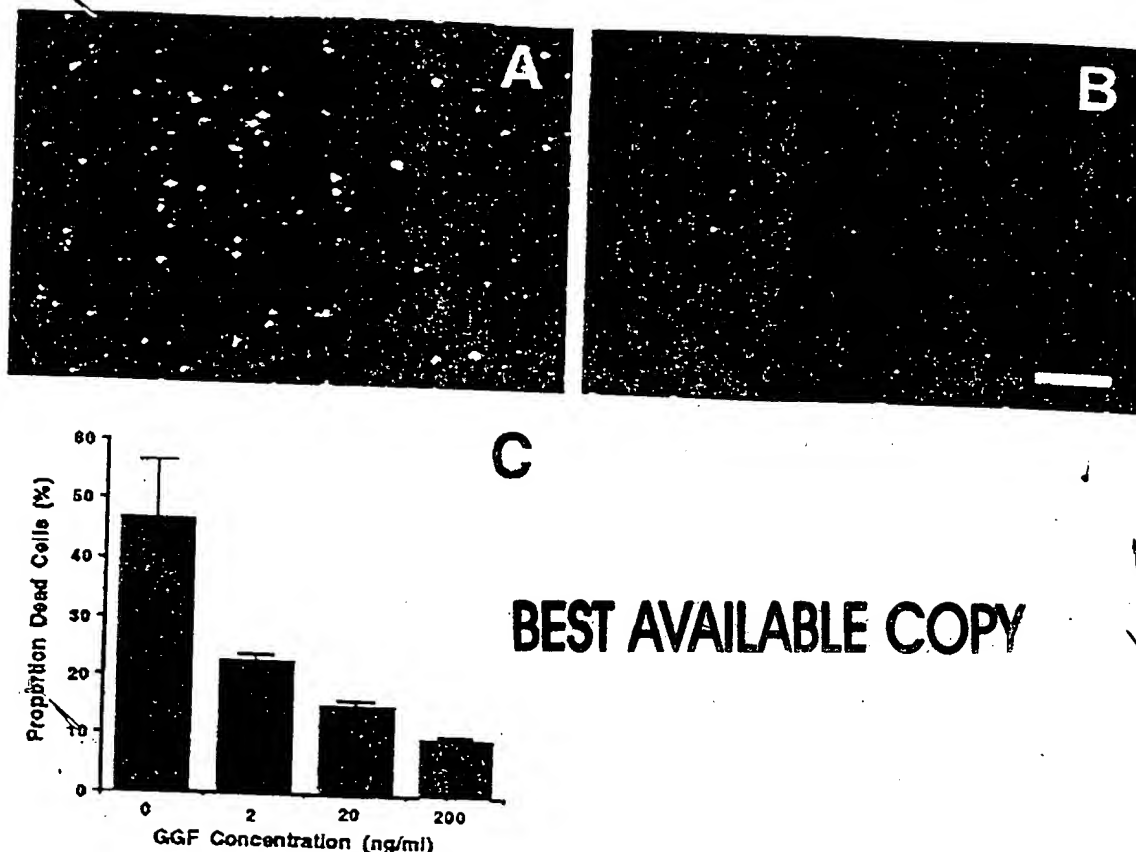


Figure 5. GGF Promotes the Survival of Oligodendrocyte Progenitors

(A) and (B) GGF maintains cell survival after serum withdrawal. Cells were switched from media containing 0.5% serum to DMEM without any serum for 12 hr without (A) or with (B) 200 ng/ml GGF. Dead cells are stained with a red nuclear stain; all other nuclei are stained blue. Representative fields are shown. Scale bar, 100 μ m. (C) Dose response of survival effects. The percentages of dead cells after switching cells to DMEM without any serum for 12 hr in the presence of increasing concentrations of GGF are shown. Each bar represents the mean \pm SEM from three experiments done in duplicate.

found that the mitogenic effects of GGF are sustained; after 3 days of GGF treatment (200 ng/ml), the labeling index was greater than 30% (Table 1) and remained comparably elevated for several weeks provided that cells continued to be passaged in the presence of GGF (data not shown). Cells treated for 3 days with GGF were also analyzed for their expression of oligodendrocyte markers. Such cells progressed to the O4+/O1- stage but were inhibited from differentiating to the O4+/O1+ stage (Table 1). For example, after 3 days of GGF, essentially all cells were O4+ and had a multipolar morphology (Figures 6A and 6B), but less than 10% of the cells

were O1+ or had acquired the complex morphology characteristic of mature oligodendrocytes (Figures 6C and 6D). This inhibition of differentiation reversed following withdrawal of GGF; we observed that 3 days after GGF withdrawal, over 50% of the cells became O1+. Thus, treatment with high doses of GGF reversibly blocks oligodendrocyte differentiation at the O4+/O1- stage.

GGF similarly inhibits differentiation of O-2A cells into type-2 astrocytes (Table 2). Cells were switched to media containing 20% FBS with or without 200 ng/ml GGF for 3 days. When grown in 20% FBS with GGF, fewer

Table 1. GGF Maintains the Proliferation and Inhibits the Differentiation of O-2A Cells Grown in DM*

Growth Condition	%O4+	%O1+	%BrDU+
-GGF	100	51.9 \pm 2.5	5.7 \pm 1.2
+GGF	100	8.1 \pm 0.6	31.3 \pm 1.3

O-2A cells in B104 conditioned media were switched to DM* with or without 200 ng/ml GGF for 3 days. Cultures were pulsed with BrDU for 4 hr and fixed and stained for O4, for O1, and for BrDU. Data shown represents the mean \pm SEM of duplicate wells from three independent experiments.

Table 2. GGF Maintains the Proliferation and Inhibits the Differentiation of O-2A Cells Grown in 20% FBS

Growth Condition	%GFAP+	%BrDU+
-GGF	48.4 \pm 4.2	5.5 \pm 1.4
+GGF	4.7 \pm 2.3	19.7 \pm 2.5

O-2A cells in B104 conditioned media were switched to media containing 20% FBS with or without 200 ng/ml GGF for 3 days. Cultures were pulsed with BrDU for 4 hr and fixed and stained for GFAP and for BrDU. Data shown represents the mean \pm SEM of duplicate wells from three independent experiments.

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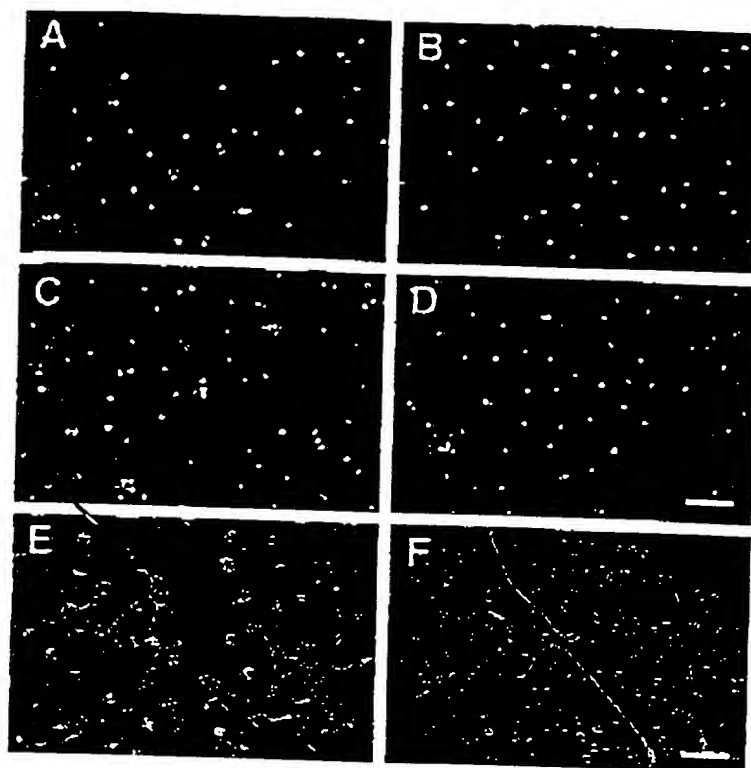


Figure 6. GGF Inhibits the Differentiation of O-2A Cells into Oligodendrocytes or Astrocytes

Cells were grown in DM* (A–D) or in 20% FBS (E and F) without (A, C, and E) or with (B, D, and F) GGF for 3 days. Cultures were fixed and stained for O4 (A and B), for O1 (C and D), or for GFAP (E and F). O4 and O1 staining was visualized with a rhodamine-conjugated antibody; GFAP with a fluorescein-conjugated antibody; in each case, nuclei were counterstained (blue). Scale bar, 100 μ m (A–D); 25 μ m (E and F).

cells expressed detectable levels of GFAP than controls. Cells that did express GFAP had a unique morphology, characterized by a few highly extended, GFAP+ processes (Figures 6E and 6F). Cells grown in 20% FBS with GGF also continued to proliferate at high levels, indicated by a labeling index of $19.7\% \pm 2.5\%$ after a 4 hr BrDU pulse compared with $5.5\% \pm 1.4\%$ in control cultures.

GGF Maintains O-2A Cells as Bipotential Cells

The ability of GGF to maintain cells as proliferating O4+/O1– progenitors suggested it might inhibit them from committing to either the oligodendrocyte or astrocyte lineages. To test this hypothesis, we grew the O-2A cells in DM* with or without GGF (200 ng/ml) for 3 days, switched all cells to media supplemented with 20% FBS without GGF for an additional 3 days, and then stained for GFAP expression. Cells grown in DM* without GGF had largely become committed to the oligodendrocyte lineage; despite switching to high serum conditions, less than 1% of cells expressed GFAP (Table 3). In contrast, when cells were grown in DM* with GGF for 3 days and then switched to 20% FBS without GGF, greater than 10% of the cells were induced to express GFAP. Thus, GGF maintained a significant number of the cells in an uncommitted bipotential state, allowing them to differentiate into astrocytes in media containing 20% FBS.

A similar experiment demonstrated that GGF also inhibits commitment to the astrocyte lineage. Cells were grown in 20% FBS for 3 days with or without GGF, and were then switched to DM* without GGF for an additional 3 days. Only about 5% of the cells grown initially without GGF expressed O1. In contrast, when cells were grown in 20% FBS with GGF and then switched to DM*, more

than 25% of the cells were induced to differentiate into O1+ oligodendrocytes (Table 2). Taken together with the studies described above, these results indicate that GGF inhibits the differentiation of O-2A cells and maintains them as proliferative bipotential cells.

Cortical Neurons Release Neuregulins

Previous studies demonstrated that cortical neurons release mitogenic factors for O-2A progenitor cells that are distinct from bFGF or PDGF (Hardy and Reynolds, 1993b). We have extended these studies by examining the effects of the CCM on O-2A cells that were allowed to differentiate in DM*. O-2A cells were grown for 12 hr or 3 days in DM* and were then treated with 33% CCM for 16 hr; BrDU was added for the last 4 hr. Analysis of BrDU incorporation showed that the CCM was mitogenic for cells in both conditions, inducing a 2–3-fold increase in cell proliferation in each case (see Figure 7).

Table 3. GGF Maintains O-2A Cells in an Uncommitted Stage

Growth Condition	DM* — FBS	FBS — DM*
	%GFAP+	%O1+
–GGF	0.4 \pm 0.1	5.9 \pm 0.2
+GGF	11.5 \pm 1.1	27.0 \pm 2.4

Left column: Cells were grown in DM* with or without GGF (200 ng/ml) for 3 days and then switched to 20% FBS for 3 days without GGF. Cultures were then stained for GFAP.

Right column: Cells were grown in 20% FBS with or without GGF (200 ng/ml) for 3 days and then switched to DM* for 3 days without GGF. Cultures were then stained for O1. Data shown represents the mean \pm SEM of duplicate wells from three independent experiments.

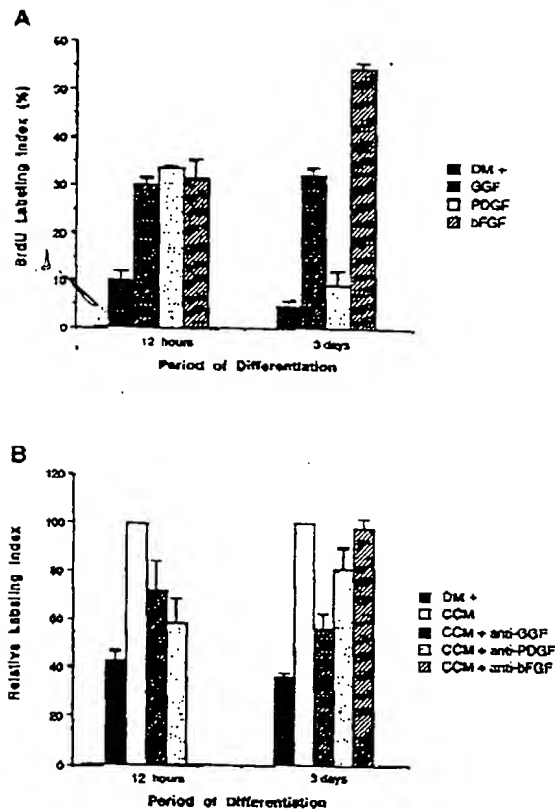


Figure 7. GGF is a Mitogen Released by Cerebral Cortical Neurons in Primary Culture

(A) PDGF, bFGF, and GGF have distinct mitogenic effects on oligodendrocyte progenitors at different stages of differentiation. Oligodendrocyte progenitors were grown in DM⁺ for 12 hr or 3 days and then treated with PDGF (10 ng/ml), bFGF (10 ng/ml), or GGF (200 ng/ml) for 16 hr. Analysis of BrdU incorporation (4 hr pulse) showed that the mitogenic response to the three growth factors changes as the cells become more differentiated. Bars represent the mean \pm SEM of duplicate wells from two to three independent experiments. (B) GGF and PDGF are released by cortical neurons. Cells were grown in DM⁺ for 3 days and then treated for 16 hr with 33% CCM that had been preincubated with an anti-GGF, an anti-PDGF, or an anti-bFGF antibody. BrdU was added during the last 4 hr of incubation. The data has been normalized to the effect of the CCM in both cases. Bars represent the mean \pm SEM of duplicate wells from three independent experiments.

Immunofluorescence analysis revealed that essentially all of the cells had advanced to the O4⁺ stage at both time points; therefore, the CCM was mitogenic for O4⁺ cells under the conditions tested. Double staining further demonstrated that all of the BrdU-positive cells were O4⁺/O1⁻ (data not shown). The CCM-treated cells had a distinctive multipolar morphology with four to ten wide flat processes consistent with our previous report (Hardy and Reynolds, 1993b) and similar to the effects we observed with GGF treatment (see Figure 1).

We next compared the mitogenic response of the oligodendrocyte progenitors grown in DM⁺ for 12 hr or for 3 days with a series of growth factors including bFGF (10 ng/ml), PDGF (10 ng/ml), and GGF (200 ng/ml). The doses of growth factors used were chosen to provide

a maximal mitogenic response based on comparative dose response curves (data not shown). As shown in Figure 7A, cells switched to DM⁺ for 12 hr (which consisted of recently differentiated O4⁺/O1⁻ cells) displayed a robust response to all three growth factors. In contrast, more mature cells that had been maintained in DM⁺ for 3 days had significantly different mitogenic response to these growth factors. In particular, the mitogenic effect of PDGF was markedly diminished on these more differentiated cells, whereas that of bFGF was increased in general agreement with previous reports (for example, see Gard and Pfeiffer, 1993). The mitogenic effect of GGF was similar for cells in both cases, indicating that it has a pattern of mitogenic activity distinct from either bFGF or PDGF.

To identify which, if any, of these polypeptide growth factors were released by cerebral neurons, we incubated the CCM with various neutralizing antibodies for 30 min prior to adding it to cells maintained in DM⁺ for either 12 hr or 3 days. Results are shown in Figure 7B. In both cases, the anti-GGF antibody significantly inhibited the CCM-induced proliferation. The inhibition of proliferation was more pronounced with the mature O4⁺ cultures (3 days in DM⁺) as compared with the newly differentiated O4⁺ cells, i.e., a 68% inhibition of the CCM effect compared with a 48% inhibition, respectively. We also observed a significant inhibition of proliferation with the anti-PDGF antibodies, particularly in the case of the newly differentiated O4⁺ cells (12 hr in DM⁺). By contrast, there were no significant effects of the anti-bFGF antibodies on the mitogenic activity of the CCM when it was tested on cells grown for 3 days in DM⁺. Taken together, these studies indicated that cortical neurons release both PDGF and GGF (or a related neuregulin isoform), with PDGF accounting for much of the mitogenic activity for early lineage cells and GGF for cells at later stages of differentiation.

Regulated Expression of Neuregulin Receptors by Cells in the Oligodendrocyte Lineage

Neuregulins bind to and signal via a subfamily of receptor tyrosine kinases that include erbB2, erbB3, and erbB4. To determine which of these receptors were expressed by cells in the oligodendrocyte lineage, we prepared lysates from O-2A cells grown in B104 conditioned media, from type-2 astrocyte-enriched cultures grown in 20% FBS, and from cells that increasingly differentiated into O4⁺/O1⁺ oligodendrocytes grown in DM⁺ for 1, 2, 3, or 4 days. Lysates were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with a panel of receptor-specific antibodies. As controls, we analyzed lysates prepared from rat Schwann cells and from newborn rat cerebrum. Results, shown in Figure 8A, demonstrate that, with the exception of Schwann cells, which are known to express only erbB2 and erbB3 proteins (Levi et al., 1995), all three receptors were expressed in each cell type and in the brain. Of note, erbB3 is a prominent neuregulin receptor protein in Schwann cells and in cells of the oligodendrocyte lineage. In contrast, erbB4 appears to predominate in the neonatal brain, which may reflect its high level expression by neurons in several regions of

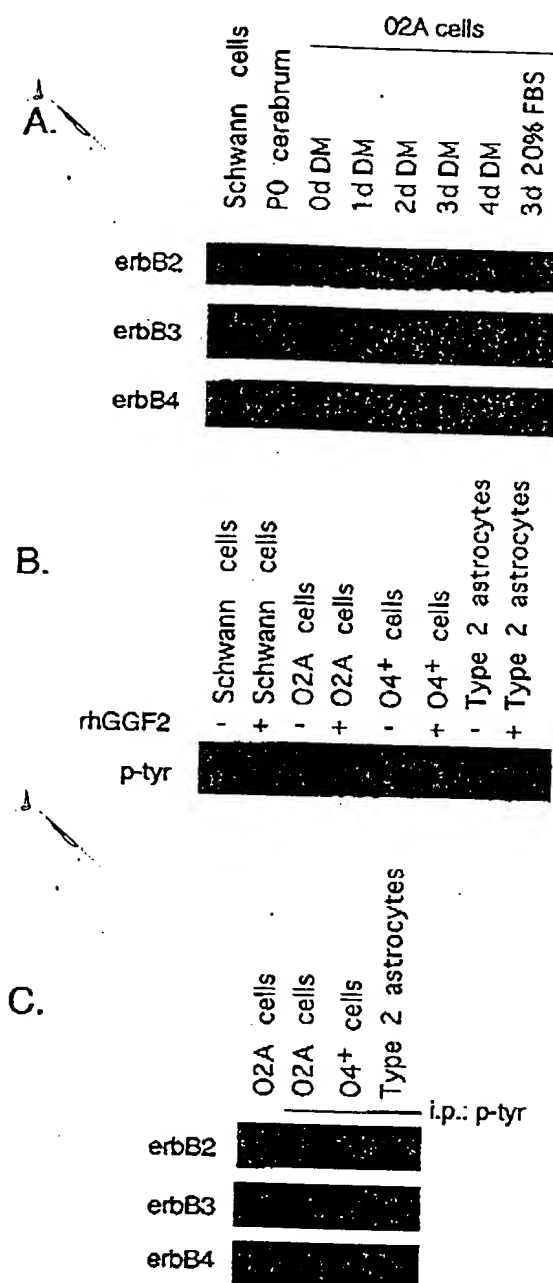


Figure 8. Expression of Receptor Isoforms by Cells in the Oligodendrocyte Lineage

(A) Expression of erbB receptors by cells in the oligodendrocyte lineage. Detergent lysates were prepared from rat Schwann cells, from P0 cerebrum, and from O-2A cells in B104 conditioned media, or grown in DM* for 1-4 days or 20% FBS for 3 days. Samples were fractionated by SDS-PAGE, blotted, and probed with antibodies specific for erbB2, erbB3, and erbB4.

(B) GGF induces phosphorylation of p185. Schwann cells, O-2A cells growing in B104 conditioned media, O4+ cells grown in DM* for 3 days, and type-2 astrocyte-enriched cultures growing in 20% FBS for 3 days were lysed directly in detergent (- lanes) or after treatment with GGF for 2.5 min (+ lanes). Detergent lysates (15 µg/lane) were subjected to SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with an anti-phosphotyrosine antibody.

the developing forebrain (characterized as tyro 2 in Lai and Lemke, 1991). Of particular interest, as cells progress along the oligodendrocyte lineage, there is a decrease in erbB4 and an increase in erbB2 expression; in some experiments, this transition was even more pronounced than is shown here. Because these cultures, even those maintained in DM* for 4 days, contain a mixture of O1- and O1+ cells, the expression of erbB4 may be even further reduced in the O1+ cells. Additional studies to address this point are in progress.

We next analyzed which proteins were phosphorylated following GGF treatment. GGF was briefly added to cultures of Schwann cells, to O-2As (in B104 conditioned media), to O4+ cells (3 days in DM*), and to type-2 astrocyte-enriched cultures (3 days in 20% FBS). Lysates were prepared, fractionated by SDS-PAGE, blotted, and probed with an anti-phosphotyrosine antibody. In each case, GGF treatment resulted in increased phosphorylation of a protein band of ~185 kDa, with the O4+ cells demonstrating the most robust response (Figure 8B). In some experiments, a protein of similar size is constitutively phosphorylated at lower levels in the O-2A and O4+ cells. To determine which of the erbB receptors were phosphorylated following GGF treatment, tyrosine phosphorylated proteins were immunoprecipitated and analyzed by immunoblotting with antibodies specific for each of the receptor proteins (Figure 8C). As a control, total lysate from the O-2A cells was also analyzed. In each case, phosphorylated erbB3 is quite prominent. Phosphorylated forms of erbB2 and erbB4 were only readily detected in the case of the O4+ cells but were also present in the case of the type-2 astrocytes with higher sample loading (data not shown).

Discussion

GGF has long been known to be a potent mitogen for Schwann cells and astrocytes (Brockes et al., 1980) but had not been recognized previously to be a mitogen for oligodendrocytes. We have demonstrated in this report that GGF is also a potent mitogen and survival factor for cells of the oligodendrocyte lineage and inhibits the differentiation of pro-oligodendrocytes. These findings, and their implications for the neuronal regulation of oligodendrocyte development, are considered below.

GGF Is Released by Neurons and Is Mitogenic for Cells at Different Stages of the Oligodendrocyte Lineage

We have found that GGF has potent mitogenic effects for pro-oligodendrocytes and oligodendrocytes and modest mitogenic effects for type-2 astrocytes. The O4+ pro-oligodendrocyte exhibits the most robust mitogenic response to GGF, which correlates well with the

(C) GGF induces phosphorylation of erbB receptors. O-2A cells in B104 conditioned media, O4+ cells grown in DM* for 3 days, and type-2 astrocyte-enriched cultures grown in 20% FBS for 3 days were treated with GGF for 2.5 min and tyrosine phosphorylated proteins were immunoprecipitated, separated by SDS-PAGE, blotted, and probed with erbB receptor-specific antibodies. A detergent lysate from untreated O-2A cells was run in the first lane as a control for antibody reactivity.

extent of receptor phosphorylation. This mitogenic activity operates directly and does not require serum cofactors, although it is significantly potentiated by low concentrations of serum. We have not examined the mitogenic effect of GGF on undifferentiated O-2A cells, because, in the culture model we have used, these cells exhibit high basal levels of proliferation as a result of expansion in the neuroblastoma conditioned media. However, addition of GGF results in the rapid tyrosine phosphorylation of a protein band of ~185 kDa for O-2A cells, suggesting that GGF may also be mitogenic for these cells. This has been confirmed in preliminary studies in which 5 ng/ml GGF resulted in a several fold increase in the proliferation of freshly plated GD3+/O4- cells (R. R., unpublished data).

Previous studies have shown that regulation of oligodendrocyte proliferation is under the control of several mitogens that operate at different stages of the oligodendrocyte lineage. Thus, PDGF acts on early progenitors, whereas during the O4+ pro-oligodendrocyte stage, cells lose their responsiveness to PDGF and become increasingly responsive to bFGF (Gard and Pfeiffer, 1990). The transition from O4+/O1- progenitors to O1+ oligodendrocytes coincides with a decrease in the mitogenic response to bFGF (Gard and Pfeiffer, 1993). Our results are consistent with these reports, as cells at an early stage of differentiation (12 hr in DM⁺) responded equally to PDGF and bFGF whereas cells at later stages of differentiation (3 days in DM⁺) were much more responsive to bFGF than to PDGF. The proliferative effects of GGF are distinct from those of PDGF and bFGF in that it is equally effective on newly differentiated O4+ cells and more mature O4+ cells. Indeed, GGF was even mitogenic for oligodendrocytes. The notion that the oligodendrocyte lineage is under the control of different mitogens is also in general agreement with the temporal pattern of expression of these growth factors in vivo. Both PDGF (Yeh et al., 1991) and the neuregulins (Corfas et al., 1995; Meyer and Birchmeier, 1994; Orr-Urtreger et al., 1993) are expressed very early during CNS development. The neuregulins, in particular, are expressed well before the oligodendrocyte progenitors are thought to arise at approximately E16 in the rat forebrain (Levine and Goldman, 1988) and they persist, at lower levels, into adulthood. By contrast, bFGF mRNA expression in the brain is first detectable postnatally and increases into adulthood (Riva and Mochetti, 1991). Taken together, these results suggest that GGF and PDGF are likely to regulate early progenitors, including O-2As and early pro-oligodendrocytes, whereas GGF and bFGF may function at later times on more differentiated pro-oligodendrocytes and oligodendrocytes. Whether GGF cooperates with these other growth factors to regulate the mitogenic response is an important question for future investigation.

Neurons are an important source of oligodendroglial mitogens (Barres and Raff, 1994), releasing soluble factors including PDGF (Dutly and Schwab, 1991; Zajicek and Compston, 1994) and bFGF (Hardy and Reynolds, 1993b; Logan and Logan, 1986) and stimulating proliferation via contact-dependent factors (Chen and DeVries, 1989; Wood and Bunge, 1986). Central neurons (Gard and Pfeiffer, 1990; Hardy and Reynolds, 1993b; Levine,

1989) and the neuroblastoma line B104 (Hunter and Botenstein, 1991) also release a soluble mitogen(s) for oligodendroglia that is distinct from PDGF and bFGF. We have found that neurons cultured from embryonic cerebral cortex release GGF into the culture media and that this GGF is mitogenic for pro-oligodendrocytes. With this report, GGF, heregulin $\beta 1$, and potentially other neuregulin isoforms expressed in the CNS (Ho et al., 1995) must now be considered likely neuronal mitogens for oligodendroglia. (In preliminary studies, we have also detected secretion of GGF by the B104 neuroblastoma line.) In agreement with this possibility, cortical neurons, which would be expected to regulate the development of forebrain-derived oligodendrocyte progenitors, express high levels of GGF during development (Chen et al., 1994). GGF and other neuregulins may also function as an axolemma-associated mitogen, as they do for Schwann cells (Rosenbaum et al., 1996; J. L. S., unpublished data). Of interest in this regard, Nordlund et al. described the partial purification of a protein of 50 kDa from brain membrane preparations that is mitogenic for both Schwann cells and oligodendrocytes (Nordlund et al., 1992) and have recently obtained evidence that it may correspond to GGF (Rosenbaum et al., 1996). Taken together, these results suggest that both soluble and membrane-associated forms of the neuregulins are likely to play an important role in regulating oligodendrocyte progenitor proliferation during development.

Based on *in situ* hybridization, neurons and cells of the SVZ appear to be major sources of neuregulins in the developing and adult CNS (Corfas et al., 1995; Orr-Urtreger et al., 1993; Vartanian et al., 1994). In addition, astrocytes also synthesize these proteins (Pinkas-Kramarski et al., 1994). While the identity of the cells expressing GGF in the SVZ has not yet been established, this region is primarily comprised of glial progenitors in the early postnatal period (Levison and Goldman, 1993). These findings suggest that both neurons and glia may be physiologic sources of neuregulins during development.

GGF Inhibits Lineage Commitment and Oligodendrocyte and Astrocyte Differentiation

Although axons are required for oligodendrocyte differentiation and myelination, tissue culture studies suggest that initially they promote proliferation and delay myelination of oligodendrocyte progenitors (Dutly and Schwab, 1991; Levine, 1989; Shaw et al., 1996; Zajicek and Compston, 1994). This inhibition of differentiation results from axolemma-associated factors (Zajicek and Compston, 1994). After this initial delay, and once an appropriate cohort of oligodendrocytes is generated, a later phase of differentiation ensues (Barres and Raff, 1994; Shaw et al., 1996; Wood and Bunge, 1991). In agreement with these initial effects of the axon, GGF promotes proliferation and significantly inhibits differentiation of oligodendrocyte progenitors beyond the O4+/O1- stage. These effects of GGF resemble those of bFGF, which, as noted above, arrests cells in the O4+/O1- stage of differentiation (McKinnon et al., 1990). Similar effects of mitogens on Schwann cell myelination (Einheber et al., 1995; Guénard et al., 1995; Morgan et al.,

1994), including GGF (Cheng and Mudge, 1996; J. L. S., unpublished data) have also been observed. These results indicate that high level expression of neuronal mitogens can promote proliferation and arrest myelination in both the CNS and PNS.

The ability of GGF to inhibit oligodendrocyte differentiation raises the question of how oligodendrocytes differentiate and myelinate axons, which are likely to express neuregulins at their cell surface. One possibility is that during CNS development, expression of neuregulin (Chen et al., 1994) or its receptors (Jin et al., 1993; Kokai et al., 1987) (or both) are down-regulated. In addition, factors released by astroglia have been reported to overcome the inhibition of differentiation of O-2A cells observed with PDGF or bFGF (Dutty and Schwab, 1991; Mayer et al., 1993) and could similarly modulate these effects of GGF *in vivo*.

The effects of GGF on type-2 astrocytes are complex. Similar to its effects in the oligodendrocyte lineage, it inhibits the differentiation of O-2A cells into type-2 astrocytes. However, it also promotes a dramatic change in the morphology of type-2 astrocytes that have already formed, resulting in the production of a few highly elongate processes (see Figure 1 and Figure 6). In addition, GGF has a modest, but significant, mitogenic effect on these cells. Some of these findings are similar to the effects of a neuregulin isoform, NDF, on type-1 astrocytes (Pinkas-Kramarski et al., 1994). These investigators found that NDF had only a modest effect on astrocyte proliferation, but promoted the formation of more extensive processes. In addition, NDF promoted astrocyte survival under serum-free conditions and increased their maturation as evidenced by up-regulation of GFAP expression, effects of GGF on type-2 astrocytes we have not analyzed.

We have further demonstrated that GGF inhibits the ability of O-2A cells to commit to either the oligodendrocyte or astrocyte lineage. In the forebrain, O-2A cells arise primarily from the SVZ and, as noted, this is a region of high level expression of GGF-related proteins. These findings raise the possibility that high level expression of GGF in this region may contribute to the high rate of proliferation and the multipotential nature characteristic of cells in this region via an autocrine or paracrine mechanism. Although cells in the postnatal SVZ are multipotential with respect to the glial lineage, giving rise to either astrocytes or oligodendrocytes, they only rarely give rise to neurons (Levison and Goldman, 1993). Whether GGF commits these cells to the glial lineage as it does for cells of the neural crest origin (Shah et al., 1994) is not yet known.

The Effects of GGF Are Not Isoform Specific

These findings contrast with a previous study (Vartanian et al., 1994), which reported that treatment of oligodendrocyte progenitors with ARIA, a related neuregulin isoform, results in increased numbers of O1+ cells from a progenitor population. In that study, treatment with ARIA did not increase the proliferation or survival of oligodendrocyte progenitors, and it was therefore suggested that ARIA promotes oligodendrocyte differentiation. As these findings are in sharp contrast with those reported

here, we investigated whether the effects of GGF were isoform specific. We analyzed the mitogenic effects of heregulin $\beta 1$, the human homolog of ARIA, and found it to be a potent mitogen, comparable in its efficacy with GGF. In addition, treatment with heregulin $\beta 1$ also appears to result in a similar loss of MBP+ cells in the treated cultures, although this effect has not been quantitated. These results indicate that the effects of GGF are not isoform specific, consistent with previous reports that the epidermal growth factor domain is sufficient to mediate the mitogenic effects of the neuregulins (Dong et al., 1995; Holmes et al., 1992; Morrissey et al., 1995). Potentially, methodologic differences between these studies may contribute to the different effects observed. First, Vartanian et al. also used supernatants from transfected COS cells as their source of ARIA, and therefore the concentration and purity of the material added to the oligodendrocyte progenitors is unknown. Their studies were also done under serum-free conditions, which we have found significantly reduced the mitogenic effect of GGF and accentuated its trophic effects. Finally, the cells used in our studies were expanded with B104 conditioned media, which might enhance their responsiveness by up-regulating oligodendroglial neuregulin receptors akin to the increase in PDGF receptors observed with bFGF treatment (McKinnon et al., 1990).

A General Role for GGF in Regulating Myelinating Glial Cell Numbers

The effects of GGF on the proliferation, survival, and maturation of oligodendrocyte progenitors are strikingly similar to its effects on cells of the Schwann cell lineage. GGF has long been known to be a potent Schwann cell mitogen and has recently been implicated in the contact-dependent stimulation of Schwann cell proliferation by neurons (Morrissey et al., 1995). In other studies, GGF has been found to inhibit the expression of myelin-specific proteins by Schwann cells (Cheng and Mudge, 1996) and their ability to myelinate axons (M. J. Hannocks, J. L. S., unpublished data). There also appears to be a transition in the expression of neuregulin receptors in the Schwann cell lineage; progenitors, but not mature Schwann cells, express erbB4 (Dong et al., 1995; Levi et al., 1995). This transition in receptor expression resembles that of the O-2A cells as they differentiate into oligodendrocytes (see Figure 8). These changes in the repertoire of receptor heterodimers expressed during the glial lineage, in turn, could be an important determinant of the cellular response, regulating receptor affinity and determining which intracellular signaling pathways are activated (Carraway and Burden, 1995; Riese et al., 1995).

Of particular note, Schwann cells (Jessen et al., 1994) and oligodendrocytes (Barres et al., 1992) are overproduced during development and undergo apoptosis owing to limiting amounts of nerve-derived survival factors. *In vitro*, neurons promote the survival of oligodendrocyte progenitors by a mechanism involving neuronal membrane-associated factors (reviewed by Barres and Raff, 1994). Recent studies have demonstrated that the neuregulins promote the survival of perinatal Schwann cells

(Trachtenberg and Thompson, 1996) and their precursors (Dong et al., 1995). The trophic effects of GGF on oligodendrocyte progenitors we have described here suggest that GGF could have a similar effect on the survival of these cells during CNS development. By promoting the survival and proliferation of both immature oligodendrocytes and Schwann cells, GGF may therefore have a crucial role during development in adjusting the final number of myelinating glial cells to the number of available axons.

In summary, we have demonstrated that GGF, a neuroregulin isoform, has potent effects on the proliferation, differentiation, and survival of oligodendrocyte progenitors. These results strongly implicate GGF in the neuronal regulation of oligodendrocyte development. Important questions remain regarding the significance of the different neuroregulin isoforms, their potential synergy with other oligodendroglial mitogens, how their activity is modulated during development, and whether they play a role in the response of the oligodendrocytes to injury, including remyelination. Analysis in coculture systems together with genetic strategies should further clarify the precise physiologic role of the neuroregulins in axon-oligodendrocyte signaling.

Experimental Procedures

Cell Culture

Primary mixed glial cell cultures, generated from 2-day-old rat forebrains were maintained in DMEM with 10% FBS for 7–10 days, after which O-2A progenitors and microglia were separated from type-1 astrocytes by shaking the cultures 250 RPM for 12 hr at 37°C (McCarthy and DeVellis, 1980). The detached O-2A cells and microglia were replated onto uncoated tissue culture dishes and left to adhere for 3 hr. This allowed the microglia to become firmly attached, whereas the O-2A progenitors were loosely attached. The O-2A cells were detached by gentle flushing, replated onto poly-L-lysine-coated dishes, and allowed to adhere overnight. The O-2A cultures were treated with 5 mM leucine methyl ester (Sigma Chemical Company, St. Louis, MO) for 15 min at room temperature to kill any remaining microglia, and then washed three times with DMEM. O-2A cells were grown in N2 media containing 10 ng/ml biotin, 30 nM T3, and 0.5% FBS (DM*) as described (McKinnon et al., 1990) and expanded by supplementing this media with either bFGF (5 ng/ml) and PDGF (10 ng/ml) or, routinely, with 30% B104 conditioned media. (B104 conditioned media was collected every 2 days from confluent cultures of this neuroblastoma line that were maintained in DM*.) Cells expanded in this manner remained undifferentiated and highly proliferative, as previously described (Bögler et al., 1990; Bottenstein and Hunter, 1990). After six passages, aliquots of 10^4 cells were prepared and frozen in liquid nitrogen. Individual aliquots were used for up to 3 months with no change in their bipolar morphology or their pattern of antigen expression. To generate cells of the oligodendrocyte lineage, this media was removed and replaced with DM* for 1–3 days. To generate type-2 astrocytes, cells were switched to DMEM supplemented with 20% FBS for 3 or more days.

Immunofluorescence Analysis

Purity of the cultures was established by immunofluorescence analysis using a series of antibodies directed against GFAP as a marker for astrocytes; OX42 monoclonal, a marker for microglia (Harlan Bioproducts for Science, Indianapolis, IN); anti-A2BS monoclonal (Boehringer Mannheim, Indianapolis, IN), a marker for O-2A progenitors; O4 and O1, which recognize early and mature oligodendrocytes, respectively (Sommer and Schachner, 1980). Cells used for immunofluorescence analysis were grown on poly-L-lysine-coated Lab-Tek eight chamber slides (Nunc). Slides were washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 15 min, and processed for immunofluorescence. Primary antibodies

were diluted in 0.1% bovine serum albumin (BSA)/PBS as follows: A2BS monoclonal, 1:100; culture supernatants of the O4 monoclonal and the O1 monoclonals were used at 1:10 and 1:1, respectively (cell lines provided by S. Pfeiffer, University of Connecticut); OX42, 1:100 (Harlan Bioproducts for Science); anti-GFAP, 1:500 (gift of A. Bignami); anti-MBP 1:500 (gift of D. Coleman). Staining with the O4 and O1 antibodies was performed on live and fixed cells as a control for specificity, with comparable results. Fluorescein- or rhodamine-conjugated secondary antibodies (Chemicon, Temecula, CA) were diluted 1:100 in 0.1% BSA/PBS. Cells stained with anti-GFAP or anti-MBP were permeabilized in 0.1% Triton X-100 for 10 min and blocked for 30 min in 0.1% BSA/PBS before incubating with secondary antibodies.

Proliferation Assays

To determine the percentage of cells synthesizing DNA, cultures were incubated for 4 hr in the presence of 10 μ M BrdU (Sigma). Cultures were then washed in PBS, fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 in PBS, and stained with the fluorescein-conjugated anti-BrdU diluted 1:10 in 0.1% BSA/PBS for 1 hr following the instructions of the manufacturer (Boehringer Mannheim). Slides were mounted in Citifluor (Citifluor Ltd., London, U.K.) containing 1 μ g/ml Hoechst nuclear dye. The labeling index, corresponding to the ratio of BrdU+ cells to total cells, was determined from photomicrographs of individual fields of BrdU-labeled and Hoechst-stained nuclei. To determine the labeling index at specific stages of differentiation, BrdU staining was combined with immunofluorescence analysis of O4 and O1 expression. Fixed cells were stained with the appropriate antibody, washed, refixed, and then permeabilized and processed for BrdU staining. A rhodamine-labeled Mu chain-specific secondary antibody (Chemicon) was included with the fluorescein anti-BrdU antibody.

Purified growth factors used in proliferation assays included PDGF AA (Sigma), bFGF (a gift of D. Rifkin, New York University Medical Center), or rhGGF2 (Cambridge Neuroscience, Cambridge, MA). Heregulin β 1 was generated in Sf9 cells infected with a recombinant baculovirus expression vector as previously described (Jo et al., 1995); controls consisted of conditioned media from Sf9 cells infected with the control vector (both gifts of S. Burden, NYU Medical Center). The Sf9 conditioned media was used at a dilution of 1:100, a concentration optimized by receptor phosphorylation studies. Cortical neuron conditioned media was prepared as previously described (Hardy and Reynolds, 1993b). Neutralizing antibodies to bFGF (a gift of D. Rifkin, NYU Medical Center), PDGF (R & D) and a rabbit polyclonal antibody raised against rhGGF2 (Cambridge Neuroscience) were used. In experiments in which these antibodies were used to characterize mitogenic activities, purified growth factors or conditioned media were preincubated with the appropriate antibody for 30 min at 37°C prior to addition to the cells. In control studies, the anti-PDGF antibody, alone or together with an anti-bFGF antibody, had no effect on the mitogenic activity of GGF whereas these antibodies completely inhibited the mitogenic activity of bFGF and PDGF, respectively (data not shown).

Survival Assays

To assess the effect of GGF on cell survival, cells growing in B104 conditioned media were changed to DM* media for 3 days. They were then switched to either N2 media or DMEM, with or without GGF, for 12 or 24 hr and stained with the Live/Dead staining kit (Molecular Probes, Inc., Eugene, OR) for 15 min following the instructions of the manufacturer. This method relies on the differential permeability of live and dead cells to a pair of fluorescent nuclear stains (when viewed with epifluorescence microscopy, the nuclei of live cells appear green whereas those of dead cells appear red). Because of fading of the green fluorescent dye, in later experiments we used the Hoechst dye (Sigma) at 1 μ g/ml after fixation as a substitute with similar results and less fading. The number of red and Hoechst dye-labeled nuclei in each field were then counted from the Ektachrome slides projected on a slide viewer; we counted $\geq 1,000$ cells per condition. In some experiments, we also used morphologic criteria, i.e., monitoring pyknotic cells under phase microscopy, and the MTT assay (Sigma).

Analysis of GGF Receptors

To analyze the expression of GGF receptors, cells at different stages of the oligodendrocyte lineage were treated with rhGGF2 at 200 ng/ml in defined media for 2.5 min at 37°C. Cells were quickly rinsed twice with ice cold PBS and lysed in cold lysis buffer containing 1% NP 40, 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 5 mM EGTA, 1 mM Na orthovanadate, 10 mM Na molybdate, 8.8 g/L Na pyrophosphate, 4 g/L NaFl, 1 mM PMSF, 10 µg/ml aprotinin, and 20 µM leupeptin. Lysates were centrifuged at 12,000 × g at 4°C for 20 min, and supernatants were reserved for analysis. We fractionated 15 µg of protein lysates by SDS-PAGE, electroblotted onto nitrocellulose, and probed with antibodies to either erbB2, erbB3, and erbB4 (Santa Cruz Biotechnology, Santa Cruz, CA) or with the anti-phosphotyrosine antibody RC 20 (Transduction Laboratories, Lexington, KY). Reacting protein bands were subsequently visualized by chemiluminescence following the instructions of the manufacturer (Amersham, Arlington Heights, IL). To analyze which receptor subunits were phosphorylated after GGF treatment, cells were lysed as above and aliquots of 150 µg were incubated with the biotinylated RC 20 anti-phosphotyrosine antibody overnight at 4°C. Immunoprecipitates were collected with streptavidin agarose beads (Pierce Chemical Company, Rockford, IL) and released by boiling in SDS sample buffer. Samples were fractionated by SDS-PAGE, electroblotted, and probed with the erbB-specific antibodies as described above.

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